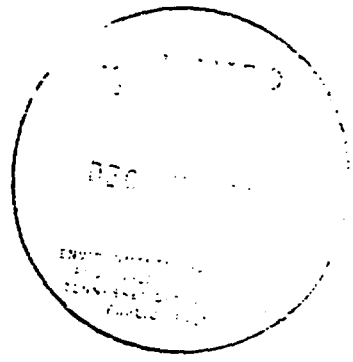
A Biological Study of Croft Springs

Division of Environmental
Laboratory Services
Tennessee Department of
Public Health



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Introduction

On September 1, 1982 a request was received from Terry K. Cothron of the Bureau of Environmental Health Services, Tennessee Department of Public Health, to make a biological study of the Croft Spring as part of a pollution evaluation. Historically, the spring has been studied at intermittent intervals since the mid 1960's. Even at this early date there were indications of its contamination.

Two or more springs are located on the Croft property. Of the two springs studied, one was included as a biological control to help delineate the problem in the other. Preliminary observations were made at the two stations September 2, 1982. A sample of a flocculent material below one of the springs was collected for microscopic analysis at this time. A second visit was made at both springs September 23, 1982 for the purpose of making a more thorough study and the collection of different types of samples, which included samples for bioassay, bacteriological, and benthic analyses. Results of the bioassays are shown in Table II, benthic analyses results are shown in Table I, and the results of the bacteriological analyses are shown in Appendices A and B. Samples for anaerobic bacterial analyses were collected October 28, 1982.

Station Locations and Descriptions

The Croft Farm is located in Metropolitan Davidson County, Tennessee. The farm extends from Trousdale Drive on the west to Nolensville Road on the east. The

larger spring (#1) is somewhat centraly located on the Croit Farm. It is approximately 200 yards North Northeast of the main residence. It surfaces at the foot of a perpendicular limestone cliff which is about 15 feet in height. The cliff is characterized by an overhanging lip at the top. Part of the flow from this spring is diverted through a cast iron pipe six inches in diameter. Smaller water lines, presently disconnected, apparently at an earlier date led to the main house as a water supply. The other spring (#2) is approximately 0.5 miles East, Northeast of the main residence. It surfaces at the base of a gentle knoll near a small tenant house. Its flow passes through a small concrete box which was uncovered.

Stream beds of the spring branches below each of the springs are made up of limestone bedrock overlaid with varying sizes of cobble. Water flowing over each stream bed is well aerated as a result of the small riffles and cobble that the streams flow over or around.

Unusual Occurrences and Observations

During the field trip September 2, 1982. a strong odor characteristic of petroleum products became apparent within 30 feet of the #1 spring. Its scent resembled diesel fuel. On the September 23, 1982 visit, most of the odor had dissipated, and was only faintly detectable even at the base of the cliff where the spring arose. On both visits, submerged dead terrestrial animals were noted to be littering the stream bed in the vicinity of the pumphouse. These dead animals included earthworms, snails, pill bugs, millipeds, and salamanders. In comparison, the control station (#2 spring) was odor-free and had no dead animals or other macroscopic indicators of pollution in its vicinity. The variation of odor intensity at #1 Croft Spring Septembr 2, 1982, and September 23, 1982, suggests the

possibility of variations in the character of the discharge from the spring. Samples for chemical determinations were not collected because of the sample overload in the organic chemistry section.

Nature of the Problem - Discussion

Radnor yards, a railroad switching yard, belongs to the Louisville and Nashville Railway Company. John P. Saad and Sons, Inc. is a waste oil processing plant. Both companies are in close proximity to each other and are located on the West side of Trousdale Drive just acorss the street from Croft Farm. In the past, both companies have been associated with pollution problems. Because of their location, either, or both companies have the potential for contaminating the #1 Croft Spring. This potential is substantiated by reports from the Divison of Water Quality files. A report dated June 19, 1968 stated:

> At the Pumphouse (#1 Croft Spring) there was a skim on the surface of the water which produced a characteristic "oil slick". This skim became widespread when the floc was distributed. The skim and odor persisted downstream at every point checked. The petroleum compound has been identified as diesel fuel by Ken Erwin of the Air Pollution Control Division of the Tennessee Department of Public Health. He analyzed a spring sample and found it to be about 0.29 (percent) diesel fuel. This diesel fuel was found to be the same as the nearby lagoon used by the Land N Railroad, by the same analysis."

On March 2, 1969, Joe Rossman, a biologist with the Cumberland Basin, collected a sample from the #1 Croft Spring that was found to contain 625 ppl chloroform, 1550 ppb carbon tetrachloride, and 1930 ppb 1-1-2 trichloroethane. In industry these are ordinary commercial solvents. However, in water supplies they are priority pollutants and recognized carcinogens. Based on a personal conversation with the chief of Laboratory Services Organic Chemistry Section, these are not compounds normally found in diesel fuel. He also stated that chloroform, carbon

tetrachloride, and 1-1-2 trichloroethane were found in drinking water supplies from some wells in Smyrna, Tennessee. It was alleged that the contaminants in the Smyrna wells had their origin from another operation in the John P. Saad and Sons, Inc. located in Rutherford County.

Observations and Results of Benthic Sampling

The stream bed and the submerged plants that grow marginally in the branch from #1 Croft Spring were overlaid and encrusted by a reddish-orange colored floc. An EPA worker stated that two days earlier (August 31, 1982) he had methodically scrubbed away the material immediately below the spring. This indicates the rapidity at which this material is being generated, and accounts for the depths of the downstream deposits. A sample of the flocculent material was collected and returned to the laboratory for analysis. A microscopic preparation was treated with In hydrochloric acid. Much of the floc dissolved in the hydrochloric acid. The treated preparation was studied at 1000 magnifications. It was found to contain two species of filamentous bacteria that resembled morphologically the iron bacteria Crenothrix sp. and Sphaerotilus sp.

Absolute identification of most bacteria is based on cultural characteristics. These characteristics are determined by the biochemical physiology of the microorganisms in question. The physiological responses of bacteria are regulated by the battery of enzymes which are characteristic for each species of bacteria. Some bacteria occurring in nature are fastidious and very difficult if not impossible to culture under laboratory conditions. For this reason, and, because of the importance of the problem, the Microbiological Section of the Division of Laboratory Services was requested to assist with the study. Personnel there are

licensed microbiologists. The results and interpretations of their findings are included in appendices A and B. Culture types and comparative quality controls were not available for their study. Their work became applied research, and is continuing. Bergey's Manual and Standard Methods were used as references for this work.

Chemical analyses of the flocculent material showed that the floc contained 136.4 ppm iron. Farther downstream, the precipitate had settled out to depths in excess of two inches. It was from this location that samples for aerobic and, at a later date, anaerobic cultures were collected. The pH of the #1 Croft Spring was 9.3. In well aerated water such as the spring branches below #1 and #2 Croft Springs, and at a weekly alkaline pH, the ferric hydroxide, when present, is precipitated in quiescent pools. The ferric hyrdoxide may be arranged in a thin colloidial layer that is noticeable because of its irridescent sheen, resembling an oil slick.[4] Two types of thin films were apparent at the #1 Spring, but not at the #2 Spring.

Examination of the stream beds below both springs, including marginal areas adjacent to the stream banks, were made. Results from this part of the investigation are summarized in Table I. Benthic invertebrates are our most reliable long-range pollution indicators for aquatic habitats. Some require as much as three years to complete the series of ecdysis that precede the various growth instars between the egg and adult phases of their life cycle. The period between instars is a time of extreme sensitivity to adverse living conditions. At #1 Spring two species of pollution tolerant protozoa, two species of filamentous bacteria, three species of resistant algae, and a bryophyte (primitive plant) that covered only the top surface of the larger stones in the stream bed, without being in actual contact with the water, comprised the observed microscopic and macroscopic

biota, with the exclusion of the microorganisms recovered by special cultures. Results of a comparable investigation at #2 Spring showed the presence of eight species of invertebrates more commonly associated with a clean water habitat. One air breathing snail (Physa sp) capable of mild organic pollution was present. Top minnows were common at this station, while none were observed at #1 Spring. Marchantia polymorpha (the primitive plant which was growing only on the top of stones out of contact with the water at #1 Spring) was widely distributed at the #2 Spring. Many of the plant thalli were in contact with the water. All the biota noted at the latter station appeared to be in good physical condition. Yentshc's [1] study of oil toxicity led him to conclude that plants are more resistant to oil toxicity than animals.

Bioassay Results

The strong petroleum-like odor noted September 2, 1982, the complete absence of aquatic invertebrates and fish, in conjunction with the occurrence of dead terrestrial aniamls in the stream at #1 Spring, indicate toxic conditions. Variation in the intensity of the petroleum-like odor on September 2 and September 23, 1982, suggest that there may be fluctuations in the stream characteristics at different times which may have influenced the results of the bioassay study.

After the first visit it was decided that a static bioassay should be conducted on the discharge from #1 Spring. The discharge from #2 Spring was used as the control. The sample and control were each set up as duplicates. The only modification made was to cover one set of the duplicates with a layer of

cellophane to prevent the escape of volatiles, if such were present; and to aerate the second set of duplicates. Both the samples and controls were used at 100 percent concentration. The study was conducted at ambient laboratory temperatures for a 96 hour period. No deaths of any of the test animals occurred during this time. The test organisms used were disease free juvenile fathead minnows. Therefore a TL_m value of the discharge from the #1 Croft Spring could not be estimated on the basis of the static bioassay. The study should be repeated as a continuous-flow bioassay because the other biological parameters are in total disagreement with the results of the static bioassay on this grab sample.

Discussion and Conclusions

The two springs studied on the Croft Farm were very different in their physical appearance, and in the type of biota that they supported. Both springs were located sufficiently close to each other to have overall general water quality characteristics that were similar, with only minor variations. Such was not the case.

Copious amounts of a reddish-orange flocculent material masked the stream bed and marginal submerged vegetation. Rheinheimer states that iron bacteria are common in freshwater springs and wells where frequently their masses can be seen with the naked eye. Some bacteria are fastidious in their physilogical requirements and may be difficult or impossible to culture under laboratory conditions. Microscopic preparations of the floc contained filamentous bacteria which morphologically resembled iron bacteria. However, final identification must rest on the biochemical reactions of the bacteria on various culture media. This is controlled by batteries of enzymes in the bacterial cells that are specific for a particular species of bacteria.

The floc was partially soluble in IN HCL and contained 136.4 mg/l iron. Iron bacteria are chemo-autotrophic. They obtain their chemical energy by reducing CO_2 and synthesizing organic material. In quiscent pools with a neutral or slightly alkaline PH a fine irridescent layer forms. This type of sheen in addition to the oil slick, was present at #1 Spring. There were no traces of the flocculent material, irridescent Fe $(QH)_3$ sheen, or oil slicks noted at #2 Spring.

The dead terrestrial organisms found below the pumphouse at #1 Spring were types of organisms known to normally live in damp surroundings. It is assumed that they inadvertantly wandered into the stream, encountered toxic conditions and were killed. This is not supported by the results of the static bioassay conducted on a grab sample of the water. However, the premise is supported by the absence of all but a few hardy pollution tolerant forms. The failure of the Bryophyte Marchantia polymorpha to grow in contact with the water at Spring #1, while growing well in contact with #2 spring water lends additional support to the possibility of poor water conditions below the pumphouse at #1 Spring.

The intensification of oil slick, after disturbing bottom sediments, correlated with fluctuations of odor intensity in the area of the pumphouse. This can be corelated with changes in stream conditions. This needs to be further verified by repeating and expanding the bioassay.

There is a need to continue the anaerobic cultures for the purpose of studying the sulfate reducing isolates. The presence of Desulfovibrio organisms can then be either confirmed or denied.

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Other participants:

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Charles Wilson	U.S. E.P.A.
Dickie Wilson	Division of Water Quality Control Cumberland Basin

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Appendix A

Croft Springs Water Examination

September 1982

On September 23, 1982 four samples of spring water were submitted to the Nashville Reference Laboratory by Mr. Harold Mullican. These samples were submitted for the purpose of determining the presence or absence of iron-fixing, sheath-forming bacteria. Four samples were submitted; two samples from the contaminated spring and two from a "clean" spring, as a control.

Samples were obtained in the following procedure:

Containers used:

Sterile, 250 ml ground glass borosilicate bottles.

Collection procedure:

Specimens were taken directly from the stream by inserting the bottle under the surface. The bottle was facing down stream as the stopper was removed and then the bottle was rotated 180° upstream, filled, and rotated downstream again. While still under water the stopper was replaced. The procedure used left a 50 ml airspace to allow for mixing.

Collected specimens were received in the laboratory about 30 minutes after collection, then refrigerated until all data had been recorded and medium set up. Samples were allowed to come to room temperature just prior to culturing.

Culturing process:

For initial growth of the suspected microorganisms we used BOD dilution water supplemented with 100 mg/l sodium lactate. Fifty milliliters (50 ml) of this medium was dispensed in French square bottles and autoclaved at 15 lbs pressure for 15 minutes.

Samples from the contaminated spring were designated as sample #'s 1 and 2; samples from the control spring were designated as #'s 3 and 4.

Specimen 1 and 2 contained rusty-orange sedimentation, whereas, specimen 3 and 4 were free of any sedimentation.

Inoculation:

Inoculation of BOD medium was as follows:

A. 25 ml sample - well mixed
B. 25 ml supernatent (heavy material all settled out)
C. 25 ml sediment (concentration of heavy material)

"B" group was duplicated simply to determine convenience of picking out of filamentous forms from an upright bottle as compared to a bottle placed on it's side to give more surface exposure.

Samples were mixed and placed in a 25°C incubator for 5 days.

At the end of 5 days all samples were examined macroscopically and microscopically for the presence of filaments within the liquid medium. No filamentous forms were observed.

With the absence of filaments in the BOD lactate broth it was decided that we would culture by streaking a loop full of the specimen.

"Isolation medium (Iron bacteria)" was used for the isolation procedure.

Each of the 16 BOD cultures were streaked for isolation and incubated overnight at 25°C. Each plate was examined for the presence of filamentous colonies. Filamentous colony types were observed on plates from all 4 samples. Suspicious colonies were transferred to Manganese (Mn) agar plates and incubated at 25°C: the isolation agar plates were reinoculated an additional 24 hours. The Mn agar is recommended for the specific differentiation of Leptothrix and Sphaerotilus. Colonies of Leptothrix are dark brown because of the presence of MnO_2, whereas colonies of Sphaerotilus are colorless. After 24 hours incubation, the Mn agar plates showed no evidence of browning. More of the Isolation medium plates exhibit filamentous colonies after additional incubation. These isolates were streaked on Mn for differentiation. No dark brown colonies were observed in this group. Both sets of the Mn agar plates were given a total of 48 hours incubation time.

The filamentous colonies observed on the Mn agar plates were picked, and transferred to Maintenance medium agar slants and incubated at 25°C for 24 hours. After incubation, the slants were placed in the refrigerator for storage until needed for further testing.

It was felt that these filamentous colonies were not the microorganisms in question. Therefore, these colonies were checked to determine if they were

actually filamentous, sheath-forming bacteria (iron bacteria) or, if they were of the pseudomonad-like type of bacteria.

Differentiation Procedure:

Eleven isolated filamentous colonies were transferred to trypticase soy broth (TSB), trypticase soy agar slants (TSA), triple sugar iron agar slants (TSI), and motility agar. TSI was used for intital determination of oxidation-fermentation reaction and for H_2S and gas production.

Sheath-forming bacteria are described as ranging from 0.35-2.4 by 3-12 um and up to 100cm whereas, Pseudomonus-like organisms generally are 0.5-1 by 1.5-4 um.

The sheathed bacteria are usually motile by means of a bundle of subpolar flagella.

Gram Morphology:

1A = Small coccoid to short, plump, vacuolated gram-negative rods. Arranged singly and no indication of filamentous forms.

1a = Small, thin gram negative rods, generally short (length typical of pseudomad-like bacteria)

1c = Gram-negative rods, short to medium some give slight diphthroid-like appearance. Arranged in singles and pairs with occasional short chaining and questionable branching. No filamentous forms.

2Ba =
Gram-negative rods, short to medium and arranged in pairs. Single forms appear as though they are being pulled apart.

2Bb =
Gram-negative rods, generally short, thin and arranged in singles and pairs. No filaments.

2c =
Small gram-negative coccoid rods; deeply stained and very few rod forms.

3A =
Basically same as "2c".

3Ba =
Basicaly same as "2c".

3Bb =
Basically same as "1A"; vacuolation not as prevalent as in 1A.

3c =
Coccoid to short, plump gram-negative rods. No filaments.

4A =
Basically same as "2c".

The morphology, as determined above, more closely resembles the pseudomonad-like bacteria than the filamentous-sheath-forming bacteria.

Iso-late	TSI* reaction	Motility	O-F Test	Biochemical Test by API-20E and other conventional tests.
1A	K/NC-	Polar tuft	Ox	oxidase test = negative; MacConkey's = growth;
1a	NC/NC	nonmotile		Fluorescein produced and API results Pseudomonas group if oxidase positive 42^{o}-
1c	NC/NC	nonmotile		
2Ba	A/A	nonmotile		
2Bb	K/NC	nonmotile	Ox	oxidase = positive; Mac. = growth; fluorescein negative API = "other Pseudomonas spp." (no number) 42^{o}+
2c	K/NC	nonmotile	Non-Ox non-Ferm	oxidase = negative; Mac. = negative; fluorescem positive API = No number = no reactions obtained 42^{o}-

Isolate	TSI* reaction	Motility	O-F Test	Biochemical Test by API-20E and other conventional tests.
3A	K/NC	Polar tuft	Ox	oxidase negative; Mac. growth; fluorescein positive; API = "other Pseudonomas Spp". if oxidase + 42^{o}-
3Ba	K/NC	Polar tuft	Ox	oxidase negative; Mac. = growth, fluorescein = positive API = No number chosen to "Other Pseudomones app." if oxidase positive 42^{o}-
3Bb	K/NC	Polar tuft		
3C	K/NC	Polar tuft		
4A	K/NC	nonmotile		

A = acid from corbonydrate fermentation

K = alkaline reaction - oxidative - type reaction

NC = no change in indicator - (phenol red)

H_2S = blackening produced within the butt of the slant

gas = indicated presence = O-agar usually split

Total reaction written as to reaction in slant and butt, i.e., short A/A butt

The iron-fixing bacteria are designated as nonfermentative bacteria in terms of their method of metabolizing glucose. The isolate giving a fermentative reaction (A/A) was not tested further. The API-2OE identification system was utilized to try to identify the isolates. All isolates were Gram-negative coccoid to short rods - no filamentous forms.

Conclusions:

With the data available from the isolation process and the Mn agar results, Mr. Mullican felt that the iron bacteria were not cultured from this sample. At this time it was decided to proceed with attempts to isolate Sulfur bacteria which are anaerobic in nature. We are presently holding the isolates obtained for future reference.

Appendix B

Croft Farm Water Specimen Work-up for Anaerobes

Three bottles were received on 10-28-82

#1 - Croft Farm Spring - 80 ft. D/S of Pumphouse

#2 - Croft Farm Spring - 40 ft. D/S of Pumphouse

#3 - Croft Farm Spring - At Pumphouse

These specimens were examined for the presence of anaerobic Desulfovibrio, a sulfate-reducing bacteria as follows:

The samples were plated when received to freshly poured agar medium as described in Standard Methods for the Examination of Water and Wastewater 15th edition, 1980, p. 783 and 918. The samples were also put into liquid sulfate-reducing medium containing sodium ascorbate and Fe (NH_4) SO_2 (same reference).

The plates were incubated anaerobically in duplicate jars at room temperature.

The liquid media was filled to the top of the tube with more media and incubated at room temperature. The tubes all showed black pigment formation after several days.

The plates were opened on 11-8-82. All plates showed mixed colony types, including several different colonies which were black when initially opened.

Five black colony types were picked to the liquid media. All except one of these showed aerobic growth, and there was no black pigment in the liquid media from any of these picked colonies.

On 11-9-82 black colonies from the duplicate set of plates were picked to fresh plating media. These were incubated anaerobically at room temperature.

On 11-15-82 black colonies from these plates were picked to liquid media. Only one tube showed subsequent blackening. This specimen also grew aerobically.

On 11-24-82 the laboratory is still working with the specimens but has not isolated Desulfovibrio organisms.

Barbie Corwin, Anaerobic Bacteriology

Table I

List of Organisms Found at Craft Springs

Classification	Spring #1	Spring #2
Chlamydobacteriales Chlamydobacteriaceae *Sphaerotilus sp.*	X	O
Crenotrichaceae Undetermined sp. Probably *Crenothrix sp.*	X	O
Ciliophora Paramecidiae *Paramecium sp.*	X	O
Vorticellidae *Vorticella sp.*	X	O
Oscillatoriales Oscillatoriaceae *Oscillatoria sp.*	X	O
Chlorophyta Zygnematales Desmidiaceae *Closterium acerosum*	X	O
Zygnemataceae *Sprirogyra sp.*	X	O
Bryophyta Hepatiaceae *Marchantia polymorpha*	X	X
Turbellaria Tricladia Planariidae *Dugesia tigrina* *Undetermined species*		X X
Crustacea Malacostraca Amphipoda Gammaridae *Gammarus sp.*	X	X

Table I (continued)

Classification	Spring #1	Spring #2
Crustacea Malacostraca Amphipoda Gammaridae Gammarus sp.	O	X
Isapoda Asellidae Lirceus sp.	O	X
Hexapoda Trichoptera Hydropsychidae Diplectrona modesta	O	X
Hexapoda Insecta Coleoptera Dytiscidae Undetermined sp.	O	X
Mollusca Gastropoda Pleuraceridae Goniobasis sp.	O	X
Physidae Physa sp.	O	X

X denotes presence of the organism
O denotes absence of the organism

Pj/5

Table II

Static Bioassay of Croft Springs

Location = Metropolitan Nashville, Davidson County

Beginning Date 9-23-82 @ 11:30 AM

Termination Date 9-27-82 @ 11:30 AM

Temperature - Ambient Laboratory

Test Organism - Pimephales promelas (juveniles)

Con.	Time in Hours	Live Organisms #1 Croft Spring A	Live Organisms #1 Croft Spring C	Live Organisms #2 Croft Spring A	Live Organisi #2 Croft Spring C
100%	0	2	2	2	2
100%	24	2	2	2	2
100%	48	2	2	2	2
100%	72	2	2	2	2
100%	96	2	2	2	2

A = Aerated

C = Covered

Tlm Based on results of this test the TLm could not be established because there were no deaths.